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APC ANTIBODIES

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TECHNICAL AREA OF THE INVENTION

The invention relates to the area of cancer diagnostics and therapeutics. More particularly, the invention relates to detection of the germline and somatic alterations of wild-type APC genes. In addition, it relates to therapeutic intervention to restore the function of APC gene product.

BACKGROUND OF THE INVENTION

According to the model of Knudson for tumorigenesis (Cancer Research, Vol. 45, p. 1482, 1985), there are tumor suppressor genes in all normal cells which, when they become non-functional due to mutation, cause neoplastic development. Evidence for this model has been found in the cases of retinoblastoma and colorectal tumors. The implicated suppressor genes in those tumors, RB, p53, DCC and MCC, were found to be deleted or altered in many cases of the tumors studied. (Hansen and Cavenee, Cancer Research, Vol. 47, pp: 5518-5527 (1987); Baker et al., Science, Vol. 244, p. 217 (1989); Fearon et al., Science, Vol. 247, p. 49 (1990); Kinzler et al. Science Vol. 251. p. 1366 (1991).)

2va. a27 In order to fully understand the pathogenesis of tumors, it will be necessary to identify the other suppressor genes that play a role in the tumorigenesis process. Prominent among these is the one(s) presumptively located at 5q21. Cytogenetic (Herrera et al., *Am J. Med. Genet.*, Vol. 25, p. 473 (1986) and linkage (Leppert et al., Science, Vol. 238, p. 1411 (1987); Bodmer et al., *Nature*, Vol. 328, p. 614 (1987)) studies have shown that this chromosome region harbors the gene responsible for familial adenomatous polyposis (FAP) and Gardner's Syndrome (GS). FAP is an autosomal-dominant, inherited disease in which affected individuals develop hundreds to thousands of adenomatous polyps, some of which progress to malignancy. GS is a variant of FAP in which desmoid tumors, osteomas and other soft tissue tumors occur together with multiple adenomas of the colon and rectum. A less severe form of polyposis has been identified in which only a few (2-40) polyps develop. This condition also is familial and is linked to the same chromosomal markers as FAP and GS (Leppert et al., *New England Journal of Medicine*, Vol. 322, pp. 904-908, 1990.) Additionally, this chromosomal region is often deleted from the adenomas (Vogelstein et al., *N. Engl. J. Med.*, Vol. 319, p. 525 (1988)) and carcinomas (Vogelstein et al., *N. Engl. J. Med.*, Vol. 319, p. 525 (1988); Solomon et al., *Nature*, Vol. 328, p. 616 (1987); Sasaki et al., *Cancer Research*, Vol. 49, p. 4402 (1989); Delattre et al., *Lancet*, Vol. 2, p. 353 (1989); and Ashton-Rickardt et al., *Oncogene*, Vol. 4, p. 1169 (1989)) of patients without FAP (sporadic tumors). Thus, a putative suppressor gene on chromosome 5q21 appears to play a role in the early stages of colorectal neoplasia in both sporadic and familial tumors.

Although the MCC gene has been identified on 5q21 as a candidate suppressor gene, it does not appear to be altered in FAP or GS patients. Thus there is a need in the art for

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investigations of this chromosomal region to identify genes and to determine if any of such genes are associated with FAP and/or GS and the process of tumorigenesis.

# SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for diagnosing and prognosing a neoplastic tissue of a human.

It is another object of the invention to provide a method of detecting genetic predisposition to cancer.

It is another object of the invention to provide a method of supplying wild-type APC gene function to a cell which has lost said gene function.

It is yet another object of the invention to provide a kit for determination of the nucleotide sequence of APC alleles by the polymerase chain reaction.

It is still another object of the invention to provide nucleic acid probes for detection of mutations in the human APC gene.

It is still another object of the invention to provide a cDNA molecule encoding the APC gene product.

It is yet another object of the invention to provide a preparation of the human APC protein.

It is another object of the invention to provide a method of screening for genetic predisposition to cancer.

It is an object of the invention to provide methods of testing therapeutic agents for the ability to suppress neoplasia.

It is still another object of the invention to provide animals carrying mutant APC alleles.

These and other objects of the invention are provided by one or more of the embodiments which are described below. In one embodiment of the present invention a method of diagnosing or prognosing a neoplastic tissue of a human is provided comprising: detecting somatic alteration of wild-type APC genes or their expression products in a sporadic colorectal cancer tissue, said alteration indicating neoplasia of the tissue.

In yet another embodiment a method is provided of detecting genetic predisposition to cancer in a human including familial adenomatous polyposis (FAP) and Gardner's Syndrome (GS), comprising: isolating a human sample selected from the group consisting of blood and fetal tissue; detecting alteration of wild-type APC gene coding sequences or their expression products from the sample, said alteration indicating genetic predisposition to cancer.

In another embodiment of the present invention a method is provided for supplying wild-type APC gene function to a cell which has lost said gene function by virtue of a mutation in the APC gene, comprising: introducing a wild-type APC gene into a cell which has lost said gene function such that said wild-type gene is expressed in the cell.

In another embodiment a method of supplying wild-type APC gene function to a cell is provided comprising: introducing a portion of a wild-type APC gene into a cell which has lost said gene function such that said portion is expressed in the cell, said portion encoding a part of the APC protein which is required for non-neoplastic growth of said cell. APC protein can also be applied to cells or administered to animals to remediate for mutant APC genes. Synthetic peptides or drugs can also be used to mimic APC function in cells which have altered APC expression.

In yet another embodiment a pair of single stranded primers is provided for determination of the nucleotide

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sequence of the APC gene by polymerase chain reaction. The sequence of said pair of single stranded DNA primers is derived from chromosome 5q band 21, said pair of primers allowing synthesis of APC gene coding sequences.

In still another embodiment of the invention a nucleic acid probe is provided which is complementary to human wild-type APC gene coding sequences and which can form mismatches with mutant APC genes, thereby allowing their detection by enzymatic or chemical cleavage or by shifts in electrophoretic mobility.

In another embodiment of the invention a method is provided for detecting the presence of a neoplastic tissue in a human. The method comprises isolating a body sample from a human; detecting in said sample alteration of a wild-type APC gene sequence or wild-type APC expression product, said alteration indicating the presence of a neoplastic tissue in the human.

In still another embodiment a cDNA molecule is provided which comprises the coding sequence of the APC gene.

In even another embodiment a preparation of the human APC protein is provided which is substantially free of other human proteins. The amino acid sequence of the protein is shown in FIG. 3 (SEQ ID NOS: 7 and 2).

In yet another embodiment of the invention a method is provided for screening for genetic predisposition to cancer, including familial adenomatous polyposis (FAP) and Gardner's Syndrome (GS), in a human. The method comprises: detecting among kindred persons the presence of a DNA polymorphism which is linked to a mutant APC allele in an individual having a genetic predisposition to cancer, said kindred being genetically related to the individual, the presence of said polymorphism suggesting a predisposition to cancer.

In another embodiment of the invention a method of testing therapeutic agents for the ability to suppress a neoplastically transformed phenotype is provided. The method comprises: applying a test substance to a cultured epithelial cell which carries a mutation in an APC allele; and determining whether said test substance suppresses the neoplastically transformed phenotype of the cell.

In another embodiment of the invention a method of testing therapeutic agents for the ability to suppress a neoplastically transformed phenotype is provided. The method comprises: administering a test substance to an animal which carries a mutant APC allele; and determining whether said test substance prevents or suppresses the growth of tumors.

In still other embodiments of the invention transgenic animals are provided. The animals carry a mutant APC allele from a second animal species or have been genetically engineered to contain an insertion mutation which disrupts an APC allele.

The present invention provides the art with the information that the APC gene, a heretofore unknown gene is, in fact, a target of mutational alterations on chromosome 5q21 and that these alterations are associated with the process of tumorigenesis. This information allows highly specific assays to be performed to assess the neoplastic status of a particular tissue or the predisposition to cancer of an individual. This invention has applicability to Familial Adenomatous Polyposis, sporadic colorectal cancers, Gardner's Syndrome, as well as the less severe familial polyposis discusses above.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows an overview of yeast artificial chromosome (YAC) contigs. Genetic distances between selected RFLP markers from within the contigs are shown in centi-Morgans.

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FIGS. 1B-1, 1B-2 and 1B-3 show a detailed map of the three central contigs. The position of the six identified genes from within the FAP region is shown; the 5' and 3' ends of the transcripts from these genes have in general not yet been isolated, as indicated by the string of dots surrounding the bars denoting the genes' positions. Selected restriction endonuclease recognition sites are indicated. B, BssH2; S, SstII; M, MluI; N, NruI.

INS DU) FIGS. 2A and 2B show the sequence of TB1 (FIG. 2A) and TB2 (FIG. 2B) genes. The cDNA sequence of the TB1 gene was determined from the analysis of 11 cDNA clones derived from normal colon and liver, as described in the text. A total of 2314 bp were contained within the overlapping cDNA clones, defining an ORF of 424 amino acids beginning at nucleotide 1. Only the predicted amino acids from the ORF are shown. The carboxy-terminal end of the ORF has apparently been identified, but the 5' end of the TB1 transcript has not yet been precisely determined.

The cDNA sequence of the TB2 gene was determined from the YS-39 clone derived as described in the text. This clone consisted of 2300 bp and defined an ORF of 185 amino acids beginning at nucleotide 1. Only the predicted amino acids are shown. The carboxy terminal end of the ORF has apparently been identified, but the 5' end of the TB2 transcript has not been precisely determined.

2mAP2) FIGS. 3A-3F show the sequence of the APC gene product (SEQ ID NO:7). The cDNA sequence was determined through the analysis of 87 cDNA clones derived from normal colon, liver, and brain. A total of 8973 bp were contained within overlapping cDNA clones, defining an ORF of 2842 amino acids. In frame stop codons surrounded this ORF, as described in the text, suggesting that the entire APC gene product was represented in the ORF illustrated. Only the predicted amino acids are shown.

FIGS. 4A and 4B show the local similarity between human APC (SEQ ID NO:2) and ral2 (SEQ ID NO:8) of yeast. FIG. 4A shows amino acids 203 to 233 of APC, and FIG. 4B shows amino acids 453 to 481 of APC. Local similarity among the APC (SEQ ID NO:2) and MCC genes (SEQ ID NO:10) genes and the m3 muscarinic acetylcholine receptor (SEQ ID NO:9) is shown. The region of the mAChR shown corresponds to that responsible for coupling the receptor to G proteins. The connecting lines indicate identities; dots indicate related amino acids residues.

FIG. 5 shows the genomic map of the 1200 kb NotI fragment at the FAP locus. The NotI fragment is shown as a bold line. Relevant parts of the deletion chromosomes from patients 3214 and 3824 are shown as stippled lines. Probes used to characterize the NotI fragment and the deletions, and three YACs from which subclones were obtained, are shown below the restriction map. The chimeric end of YAC 183H12 is indicated by a dotted line. The orientation and approximate position of MCC are indicated above the map.

FIG. 6A-6D show the DNA sequence (SEQ ID NO:3) and predicted amino acid sequence of DP1 (TB2) (SEQ ID NO:4). The nucleotide numbering begins at the most 5' nucleotide isolated. A proposed initiation methionine (base 77) is indicated in bold type. The entire coding sequence is presented.

FIG. 7A, FIG. 7B-1, and FIG. 7B-2 show the arrangement of exons in DP2.5 (APC). (A) Exon 9 corresponds to nucleotides 933-1312; exon 9a corresponds to nucleotides 1236-1312. The stop codon in the cDNA is at nucleotide 8535. (B) Partial intronic sequence surrounding each exon is shown (SEQ ID NO: 11-38). 5' intron sequences of exons 2,

3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 are shown in SEQ ID NOS: 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, respectively. 3' intron sequences of exons 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 are shown in SEQ ID NOS: 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 5 respectively.

#### DETAILED DESCRIPTION

It is a discovery of the present invention that mutational events associated with tumorigenesis occur in a previously unknown gene on chromosome 5q named here the APC (Adenomatous Polyposis Coil) gene. Although it was previously known that deletion of alleles on chromosome 5q were common in certain types of cancers, it was not known that a target gene of these deletions was the APC gene. Further it was not known that other types of mutational events in the APC gene are also associated with cancers. The mutations of the APC gene can involve gross rearrangements, such as insertions and deletions. Point mutations have also been observed.

According to the diagnostic and prognostic method of the present invention, alteration of the wild-type APC gene is detected. "Alteration of a wild-type gene" according to the present invention encompasses all forms of mutations—including deletions. The alteration may be due to either rearrangements such as insertions, inversions, and deletions, or to point mutations. Deletions may be of the entire gene or only a portion of the gene. Somatic mutations are those which occur only in certain tissues, e.g., in the tumor tissue, and are not inherited in the germline. Germline mutations can be found in any of a body's tissues. If only a single allele is somatically mutated, an early neoplastic state is indicated. However, if both alleles are mutated then a late neoplastic state is indicated. The finding of APC mutations thus provides both diagnostic and prognostic information. An APC allele which is not deleted (e.g., that on the sister chromosome to a chromosome carrying an APC deletion) can be screened for other mutations, such as insertions, small deletions, and point mutations. It is believed that many mutations found in tumor tissues will be those leading to decreased expression of the APC gene product. However, mutations leading to non-functional gene products would also lead to a cancerous state. Point mutational events may occur in regulatory regions, such as in the promoter of the gene, leading to loss or diminution of expression of the mRNA. Point mutations may also abolish proper RNA processing, leading to loss of expression of the APC gene product.

In order to detect the alteration of the wild-type APC gene in a tissue, it is helpful to isolate the tissue free from surrounding normal tissues. Means for enriching a tissue preparation for tumor cells are known in the art. For example, the tissue may be isolated from paraffin or cryostat sections. Cancer cells may also be separated from normal cells by flow cytometry. These as well as other techniques for separating tumor from normal cells are well known in the art. If the tumor tissue is highly contaminated with normal cells, detection of mutations is more difficult.

Detection of point mutations may be accomplished by molecular cloning of the APC allele (or alleles) and sequencing that allele(s) using techniques well known in the art. Alternatively, the polymerase chain reaction (PCR) can be used to amplify gene sequences directly from a genomic DNA preparation from the tumor tissue. The DNA sequence of the amplified sequences can then be determined. The polymerase chain reaction itself is well known in the art.

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See, e.g., Saiki et al., *Science*, Vol. 239, p. 487, 1988; U.S. Pat. No. 4,683,203; and U.S. Pat. No. 4,683,195. Specific primers which can be used in order to amplify the gene will be discussed in more detail below. The ligase chain reaction, which is known in the art, can also be used to amplify APC sequences. See Wu et al., *Genomics*, Vol. 4, pp. 560-569 (1989). In addition, a technique known as allele specific PCR can be used. (See Ruano and Kidd, *Nucleic Acids Research*, Vol. 17, p. 8392, 1989.) According to this technique, primers are used which hybridize at their 3' ends to a particular APC mutation. If the particular APC mutation is not present, an amplification product is not observed. Amplification Refractory Mutation System (ARMS) can also be used as disclosed in European Patent Application Publication No. 0332435 and in Newton et al., *Nucleic Acids Research*, Vol. 17, p.7, 1989. Insertions and deletions of genes can also be detected by cloning, sequencing and amplification. In addition, restriction fragment length polymorphism (RFLP) probes for the gene or surrounding marker genes can be used to score alteration of an allele or an insertion in a polymorphic fragment. Such a method is particularly useful for screening among kindred persons of an affected individual for the presence of the APC mutation found in that individual. Single stranded conformation polymorphism (SSCP) analysis can also be used to detect base change variants of an allele. (Orita et al., *Proc. Natl. Acad. Sci. USA* Vol. 86, pp. 2766-2770, 1989, and *Genomics*, Vol. 5, pp. 874-879, 1989.) Other techniques for detecting insertions and deletions as are known in the art can be used.

*In APP* Alteration of wild-type genes can also be detected on the basis of the alteration of a wild-type expression product of the gene. Such expression products include both the APC mRNA as well as the APC protein product. The sequences of these products are shown in FIG. 3. Point mutations may be detected by amplifying and sequencing the mRNA or via molecular cloning of cDNA made from the mRNA. The sequence of the cloned cDNA can be determined using DNA sequencing techniques which are well known in the art. The cDNA can also be sequenced via the polymerase chain reaction (PCR) which will be discussed in more detail below.

*Ins. 94* [Mismatches, according to the present invention are hybridized nucleic acid duplexes which are not 100% homologous. The lack of total homology may be due to deletions, insertions, inversions, substitutions or frameshift mutations. Mismatch detection can be used to detect point mutations in the gene or its mRNA product. While these techniques are less sensitive than sequencing, they are simpler to perform on a large number of tumor samples. An example of a mismatch cleavage technique is the RNase protection method, which is described in detail in Winter et al., *Proc. Natl. Acad. Sci. USA*, Vol. 82, p. 7575, 1985 and Meyers et al., *Science*, Vol. 230, p. 1242, 1985. In the practice of the present invention the method involves the use of a labeled riboprobe which is complementary to the human wild-type APC gene coding sequence. The riboprobe and either mRNA or DNA isolated from the tumor tissue are annealed (hybridized) together and subsequently digested with the enzyme RNase A which is able to detect some mismatches in a duplex RNA structure. If a mismatch is detected by RNase A, it cleaves at the site of the mismatch. Thus, when the annealed RNA preparation is separated on an electrophoretic gel matrix, if a mismatch has been detected and cleaved by RNase A, an RNA product will be seen which is smaller than the full-length duplex RNA for the riboprobe and the mRNA or DNA. The riboprobe need not be the full length of the APC mRNA or gene but can be a

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segment of either. If the riboprobe comprises only a segment of the APC mRNA or gene it will be desirable to use a number of these probes to screen the whole mRNA sequence for mismatches.

In similar fashion, DNA probes can be used to detect mismatches, through enzymatic or chemical cleavage. See, e.g., Cotton et al., *Proc. Natl. Acad. Sci. USA*, Vol. 85, 4397, 1988; and Shenk et al., *Proc. Natl. Acad. Sci. USA*, Vol. 72, p. 989, 1975. Alternatively, mismatches can be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes. See, e.g., Cariello, *Human Genetics*, Vol. 42, p. 726, 1988. With either riboprobes or DNA probes, the cellular mRNA or DNA which might contain a mutation can be amplified using PCR (see below) before hybridization. Changes in DNA of the APC gene can also be detected using Southern hybridization, especially if the changes are gross rearrangements, such as deletions and insertions.

DNA sequences of the APC gene which have been amplified by use of polymerase chain reaction may also be screened using allele-specific probes. These probes are nucleic acid oligomers, each of which contains a region of the APC gene sequence harboring a known mutation. For example, one oligomer may be about 30 nucleotides in length, corresponding to a portion of the APC gene sequence. By use of a battery of such allele-specific probes, PCR amplification products can be screened to identify the presence of a previously identified mutation in the APC gene. Hybridization of allele-specific probes with amplified APC sequences can be performed, for example, on a nylon filter. Hybridization to a particular probe under stringent hybridization conditions indicates the presence of the same mutation in the tumor tissue as in the allele-specific probe.

Alteration of APC mRNA expression can be detected by any technique known in the art. These include Northern blot analysis, PCR amplification and RNase protection. Diminished mRNA expression indicates an alteration of the wild-type APC gene. Alteration of wild-type APC genes can also be detected by screening for alteration of wild-type APC protein. For example, monoclonal antibodies immunoreactive with APC can be used to screen a tissue. Lack of cognate antigen would indicate an APC mutation. Antibodies specific for products of mutant alleles could also be used to detect mutant APC gene product. Such immunological assays can be done in any convenient format known in the art. These include Western blots, immunohistochemical assays and ELISA assays. Any means for detecting an altered APC protein can be used to detect alteration of wild-type APC genes. Functional assays can be used, such as protein binding determinations. For example, it is believed that APC protein oligomerizes to itself and/or MCC protein or binds to a G protein. Thus, an assay for the ability to bind to wild type APC or MCC protein or that G protein can be employed. In addition, assays can be used which detect APC biochemical function. It is believed that APC is involved in phospholipid metabolism. Thus, assaying the enzymatic products of the involved phospholipid metabolic pathway can be used to determine APC activity. Finding a mutant APC gene product indicates alteration of a wild-type APC gene.

Mutant APC genes or gene products can also be detected in other human body samples, such as, serum, stool, urine and sputum. The same techniques discussed above for detection of mutant APC genes or gene products in tissues can be applied to other body samples. Cancer cells are sloughed off from tumors and appear in such body samples. In addition, the APC gene product itself may be secreted into

the extracellular space and found in these body samples even in the absence of cancer cells. By screening such body samples, a simple early diagnosis can be achieved for many types of cancers. In addition, the progress of chemotherapy or radiotherapy can be monitored more easily by testing such body samples for mutant APC genes or gene products.

The methods of diagnosis of the present invention are applicable to any tumor in which APC has a role in tumorigenesis. Deletions of chromosome arm 5q have been observed in tumors of lung, breast, colon, rectum, bladder, liver, sarcomas, stomach and prostate, as well as in leukemias and lymphomas. Thus these are likely to be tumors in which APC has a role. The diagnostic method of the present invention is useful for clinicians so that they can decide upon an appropriate course of treatment. For example, a tumor displaying alteration of both APC alleles might suggest a more aggressive therapeutic regimen than a tumor displaying alteration of only one APC allele.

The primer pairs of the present invention are useful for determination of the nucleotide sequence of a particular APC allele using the polymerase chain reaction. The pairs of single stranded DNA primers can be annealed to sequences within or surrounding the APC gene on chromosome 5q in order to prime amplifying DNA synthesis of the APC gene itself. A complete set of these primers allows synthesis of all of the nucleotides of the APC gene coding sequences, i.e., the exons. The set of primers preferably allows synthesis of both intron and exon sequences. Allele specific primers can also be used. Such primers anneal only to particular APC mutant alleles, and thus will only amplify a product in the presence of the mutant allele as a template.

In order to facilitate subsequent cloning of amplified sequences, primers may have restriction enzyme site sequences appended to their 5' ends. Thus, all nucleotides of the primers are derived from APC sequences or sequences adjacent to APC except the few nucleotides necessary to form a restriction enzyme site. Such enzymes and sites are well known in the art. The primers themselves can be synthesized using techniques which are well known in the art. Generally, the primers can be made using oligonucleotide synthesizing machines which are commercially available. Given the sequence of the APC open reading frame shown in FIG. 3 (SEQ ID NO:1), design of particular primers is well within the skill of the art.

The nucleic acid probes provided by the present invention are useful for a number of purposes. They can be used in Southern hybridization to genomic DNA and in the RNase protection method for detecting point mutations already discussed above. The probes can be used to detect PCR amplification products. They may also be used to detect mismatches with the APC gene or mRNA using other techniques. Mismatches can be detected using either enzymes (e.g., S1 nuclease), chemicals (e.g., hydroxylamine or osmium tetroxide and piperidine), or changes in electrophoretic mobility of mismatched hybrids as compared to totally matched hybrids. These techniques are known in the art. See, Cotton, supra, Shenk, supra, Myers, supra, Winter, supra, and Novack et al., Proc. Natl. Acad. Sci. USA, Vol. 83, p. 586, 1986. Generally, the probes are complementary to APC gene coding sequences, although probes to certain introns are also contemplated. An entire battery of nucleic acid probes is used to compose a kit for detecting alteration of wild-type APC genes. The kit allows for hybridization to the entire APC gene. The probes may overlap with each other or be contiguous.

If a riboprobe is used to detect mismatches with mRNA, it is complementary to the mRNA of the human wild-type



APC gene. The riboprobe thus is an anti-sense probe in that it does not code for the APC protein because it is of the opposite polarity to the sense strand. The riboprobe generally will be labeled with a radioactive, colorimetric, or fluorometric material, which can be accomplished by any means known in the art. If the riboprobe is used to detect mismatches with DNA it can be of either polarity, sense or anti-sense. Similarly, DNA probes also may be used to detect mismatches.

Nucleic acid probes may also be complementary to mutant alleles of the APC gene. These are useful to detect similar mutations in other patients on the basis of hybridization rather than mismatches. These are discussed above and referred to as allele-specific probes. As mentioned above, the APC probes can also be used in Southern hybridizations to genomic DNA to detect gross chromosomal changes such as deletions and insertions. The probes can also be used to select cDNA clones of APC genes from tumor and normal tissues. In addition, the probes can be used to detect APC mRNA in tissues to determine if expression is diminished as a result of alteration of wild-type APC genes.

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According to the present invention a method is also provided of supplying wild-type APC function to a cell which carries mutant APC alleles. Supplying such function should suppress neoplastic growth of the recipient cells. The wild-type APC gene or a part of the gene may be introduced into the cell in a vector such that the gene remains extrachromosomal. In such a situation the gene will be expressed by the cell from the extrachromosomal location. If a gene portion is introduced and expressed in a cell carrying a mutant APC allele, the gene portion should encode a part of the APC protein which is required for non-neoplastic growth of the cell. More preferred is the situation where the wild-type APC gene or a part of it is introduced into the mutant cell in such a way that it recombines with the endogenous mutant APC gene present in the cell. Such recombination requires a double recombination event which results in the correction of the APC gene mutation. Vectors for introduction of genes both for recombination and for extrachromosomal maintenance are known in the art and any suitable vector may be used. Methods for introducing DNA into cells such as electroporation, calcium phosphate co-precipitation and viral transduction are known in the art and the choice of method is within the competence of the routineer. Cells transformed with the wild-type APC gene can be used as model systems to study cancer remission and drug treatments which promote such remission.

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[Similarly, cells and animals which carry a mutant APC allele can be used as model systems to study and test for substances which have potential as therapeutic agents. The cells are typically cultured epithelial cells. These may be isolated from individuals with APC mutations, either somatic or germline. Alternatively, the cell line can be engineered to carry the mutation in the APC allele. After a test substance is applied to the cells, the neoplastically transformed phenotype of the cell will be determined. Any trait of neoplastically transformed cells can be assessed, including anchorage-independent growth, tumorigenicity in nude mice, invasiveness of cells, and growth factor dependence. Assays for each of these traits are known in the art.]

Animals for testing therapeutic agents can be selected after mutagenesis of whole animals or after treatment of germline cells or zygotes. Such treatments include insertion of mutant APC alleles, usually from a second animal species, as well as insertion of disrupted homologous genes. Alternatively, the endogenous APC gene(s) of the animals may be disrupted by insertion or deletion mutation. After test

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Active APC molecules can be introduced into cells by microinjection or by use of liposomes, for example. Alternatively, some such active molecules may be taken up by cells, actively or by diffusion. Extracellular application of APC gene product may be sufficient to affect tumor growth. Supply of molecules with APC activity should lead to a partial reversal of the neoplastic state. Other molecules with APC activity may also be used to effect such a reversal, for example peptides, drugs, or organic compounds.

45      Predisposition to cancers as in FAP and GS can be  
ascertained by testing any tissue of a human for mutations of  
the APC gene. For example, a person who has inherited a  
germline APC mutation would be prone to develop cancers.  
50      This can be determined by testing DNA from any tissue of  
the person's body. Most simply, blood can be drawn and  
DNA extracted from the cells of the blood. In addition,  
prenatal diagnosis can be accomplished by testing fetal cells,  
placental cells, or amniotic fluid for mutations of the APC  
55      gene. Alteration of a wild-type APC allele, whether for  
example, by point mutation or by deletion, can be detected  
by any of the means discussed above.

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A short region of homology has been identified between APC and the human m3 muscarinic acetylcholine receptor

(mAChR). This homology was largely confined to 29 residues in which 6 out of 7 amino acids (EL(GorA)GLQA) were identical (See FIG. 4 (SEQ ID NO: 9)). Initially, it was not known whether this homology was significant, because many other proteins had higher levels of global homology (though few had six out of seven contiguous amino acids in common). However, a study on the sequence elements controlling G protein activation by mAChR subtypes (Lechleiter et al., EMBO J., p. 4381 (1990)) has shown that a 21 amino acid region from the m3 mAChR completely mediated G protein specificity when substituted for the 21 amino acids of m2 mAChR at the analogous protein position. These 21 residues overlap the 19 amino acid homology between APC and m3 mAChR.]

This connection between APC and the G protein activating region of mAChR is intriguing in light of previous investigations relating G proteins to cancer. For example, the RAS oncogenes, which are often mutated in colorectal cancers (Vogelstein, et al., N. Engl. J. Med., Vol. 319, p. 525 (1988); Bos et al., Nature Vol. 327, p. 293 (1987)), are members of the (1 protein family (Bourne, et al, Nature, Vol. 348, p. 125 (1990)) as is an in vitro transformation suppressor (Noda et al., Proc. Natl. Acad. Sci. USA, Vol. 86, p. 162 (1989)) and genes mutated in hormone producing tumors (Candis et al., Nature, Vol. 340, p. 692 (1989); Lyons et al., Science, Vol. 249, p. 655 (1990)). Additionally, the gene responsible for neurofibromatosis (presumably a tumor suppressor gene) has been shown to activate the GTPase activity of RAS (Xu et al., Cell, Vol. 63, p. 835 (1990); Martin et al., Cell, Vol. 63, p. 843 (1990); Ballester et al., Cell, Vol. 63, p. 851 (1990)). Another interesting link between G proteins and colon cancer involves the drug sulindac. This agent has been shown to inhibit the growth of benign colon tumors in patients with FAP, presumably by virtue of its activity as a cyclooxygenase inhibitor (Waddell et al., J. Surg. Oncology 24(1), 83 (1983); Wadell, et al., Am. J. Surg., 157(1), 175 (1989); Charneau et al., Gastroenterologie Clinique at Biologique 14(2), 153 (1990)). Cyclooxygenase is required to convert arachidonic acid to prostaglandins and other biologically active molecules. G proteins are known to regulate phospholipase A2 activity, which generates arachidonic acid from phospholipids (Role et al., Proc. Natl. Acad. Sci. USA, Vol. 84, p. 3623 (1987); Kurachi et al., Nature, Vol. 337, 12 555 (1989)). Therefore we propose that wild-type APC protein functions by interacting with a G protein and is involved in phospholipid metabolism.

The following are provided for exemplification purposes only and are not intended to limit the scope of the invention which has been described in broad terms above.

#### EXAMPLE 1

21.97 [This example demonstrates the isolation of a 5.5 Mb region of human DNA linked to the FAP locus. Six genes are identified in this region, all of which are expressed in normal colon cells and in colorectal, lung, ad bladder tumors.]

The cosmid markers YN5.64 and YN5.48 have previously been shown to delimit an 8 cM region containing the locus for FAP (Nakamura et al., Am. J. Hum. Genet. Vol. 43, p. 638 (1988)). Further linkage and pulse-field gel electrophoresis (PFGE) analysis with additional markers has shown that the FAP locus is contained within a 4 cM region bordered by cosmids EF5.44 and L5.99. In order to isolate clones representing a significant portion of this locus, a yeast artificial chromosome (YAC) library was screened with various 5q21 markers. Twenty-one YAC clones, distributed

within six contigs and including 5.5 Mb from the region between YN5.64 and YN5.48, were obtained (FIG. 1A).

Three contigs encompassing approximately 4 Mb were contained within the central portion of this region. The YAC's constituting these contigs, together with the markers used for their isolation and orientations, are shown in FIG. 1. These YAC contigs were obtained in the following way. To initiate each contig, the sequence of a genomic marker cloned from chromosome 5q21 was determined and used to design primers for PCR. PCR was then carried out on pools of YAC clones distributed in microtiter trays as previously described (Anand et al., *Nucleic Acids Research*, Vol. 18, p. 1951 (1990)). Individual YAC clones from the positive pools were identified by further PCR or hybridization based assays, and the YAC sizes were determined by PFGE.]

To extend the areas covered by the original YAC clones, "chromosomal walking" was performed. For this purpose, YAC termini were isolated by a PCR based method and sequenced (Riley et al., *Nucleic Acids Research*, Vol. 18, p. 2887 (1990)). PCR primers based on these sequences were then used to rescreen the YAC library. For example, the sequence from an intron of the FER gene (Hao et al., *Mol. Cell. Biol.*, Vol. 9, p. 1587 (1989)) was used to design PCR primers for isolation of the 28EC1 and 5EH8 YACs. The termini of the 28EC1 YAC were sequenced to derive markers RHE28 and LHE28, respectively. The sequences of these two markers were then used to isolate YAC clones 15CH12 (from RHE28) and 40CF1 and 29EF1 (from LHE28). These five YAC's formed a contig encompassing 1200 kb (contig 1, FIG. 1B).]

Similarly, contig 2 was initiated using cosmid N5.66 sequences, and contig 3 was initiated using sequences both from the MCC gene and from cosmid EF5.44. A walk in the telomeric direction from YAC 14FH1 and a walk in the opposite direction from YAC 39GG3 allowed connection of the initial contig 3 clones through YAC 37HG4 (FIG. 1B). YAC37HG4 was deposited at the National Collection of Industrial and Marine Bacteria (NCIMB), P.O. Box 31, 23 St. Machar Drive, Aberdeen AB2 1RY, Scotland, under Accession No. 40353 on Dec. 17, 1990.

Multipoint linkage analysis with the various markers used to define the contigs, combined with PFGE analysis, showed that contigs 1 and 2 were centromeric to contig 3. These contigs were used as tools to orient and/or identify genes which might be responsible for FAP. Six genes were found to lie within this cluster of YAC's, as follows:]

Contig #1: FER—The FER gene was discovered through its homology to the viral oncogene ABL (Hao et al., *supra*). It has an intrinsic tyrosine kinase activity, and in situ hybridization with an FER probe showed that the gene was located at 5q11-23 (Morris et al., *Cytogenet. Cell. Genet.*, Vol. 53, p. 4, (1990)). Because of the potential role of this oncogene-related gene in neoplasia, we decided to evaluate it further with regards to the FAP locus. A human genomic clone from FER was isolated (MF 2.3) and used to define a restriction fragment length polymorphism (RFLP), and the RFLP in turn used to map FER by linkage analysis using a panel of three generation families. This showed that FER was very tightly linked to previously defined polymorphic markers for the FAP locus. The genetic mapping of FER was complemented by physical mapping using the YAC clones derived from FER sequences (FIG. 1B). Analysis of YAC contig 1 showed that FER was within 600 kb of cosmid marker M5.28, which maps to within 1.5 Mb of cosmid L5.99 by PFGE of human genomic DNA. Thus, the YAC mapping results were consistent with the FER linkage data and PFGE analyses.

In. Or. 11) Contig 2: TB1—TB1 was identified through a cross-  
 hybridization approach. Exons of genes are often evolution-  
 arily conserved while introns and intergenic regions are  
 much less conserved. Thus, it a human probe cross-  
 hybridizes strongly to the DNA from non-primate species,  
 there is a reasonable chance that it contains exon sequences.  
 Subclones of the cosmids shown in FIG. 1 were used to  
 screen Southern blots containing rodent DNA samples. A  
 subclone of cosmid N5.66 (p 5.66-4) was shown to strongly  
 hybridize to rodent DNA, and this clone was used to screen  
 cDNA libraries derived from normal adult colon and fetal  
 liver. The ends of the initial cDNA clones obtained in this  
 screen were then used to extend the cDNA sequence.  
 Eventually, 11 cDNA clones were isolated, covering 2314  
 bp. The gene detected by these clones was named TB1.  
 Sequence analysis of the overlapping clones revealed an  
 open reading frame (ORF) that extended for 1302 bp starting  
 from the most 5' sequence data obtained (FIG. 2A). If this  
 entire open reading frame were translated, it would encode  
 434 amino acids (SEQ ID NO:5). The product of this gene  
 was not globally homologous to any other sequence in the  
 current database but showed two significant local similari-  
 ties to a family of ADP, ATP carrier/translocator proteins and  
 mitochondrial brown fat uncoupling proteins which are  
 widely distributed from yeast to mammals. These conserved  
 regions of TB1 (underlined in FIG. 2A) may define a  
 predictive motif for this sequence family. In addition, TB1  
 appeared to contain a signal peptide (or mitochondrial  
 targeting sequence) as well as at least 7 transmembrane  
 domains.

Contig 3: MCC, TB2, SRP and APC—The MCC gene  
 was also discovered through a cross-hybridization approach,  
 as described previously (Kinzler et al., Science Vol. 251, p.  
 1366 (1991)). The MCC gene was considered a candidate  
 for causing FAP by virtue of its tight genetic linkage to FAP  
 susceptibility and its somatic mutation in sporadic colorectal  
 carcinomas. However, mapping experiments suggested that  
 the coding region of MCC was approximately 50 kb proximal  
 to the centromeric end of a 200 kb deletion found in an  
 FAP patient. MCC cDNA probes detected a 10 kb mRNA  
 transcript on Northern blot analysis of which 4151 bp,  
 including the entire open reading frame, have been cloned.  
 Although the 3' non-translated portion or an alternatively  
 spliced form of MCC might have extended into this deletion,  
 it was possible that the deletion did not affect the MCC gene  
 product. We therefore used MCC sequences to initiate a  
 YAC contig, and subsequently used the YAC clones to  
 identify genes 50 to 250 kb distal to MCC that might be  
 contained within the deletion.

In a first approach, the insert from YAC24ED6 (FIG. 1B) was  
 radiolabelled and hybridized to a cDNA library from  
 normal colon. One of the cDNA clones (YS39) identified in  
 this manner detected a 3.1 kb mRNA transcript when used  
 as a probe for Northern blot hybridization. Sequence analy-  
 sis of the YS39 clone revealed that it encompassed 2283  
 nucleotides and contained an ORF that extended for 555 bp  
 from the most 5' sequence data obtained. If all of this ORF  
 were translated, it would encode 185 amino acids (SEQ ID  
 NO:6) (FIG. 2B). The gene detected by YS39 was named  
 TB2. Searches of nucleotide and protein databases revealed  
 that the TB2 gene was not identical to any previously  
 reported sequences nor were there any striking similarities.

Another clone (YS11) identified through the YAC 24ED6  
 screen appeared to contain portions of two distinct genes.  
 Sequences from one end of YS11 were identical to at least  
 180 bp of the signal recognition particle protein SRP19  
 (Lingelbach et al. Nucleic Acids Research, Vol. 16, p. 9431

(1988). A second ORF, from the opposite end of clone YS11, proved to be identical to 78 bp of a novel gene which was independently identified through a second YAC-based approach. For the latter, DNA from yeast cells containing YAC 14FH1 (FIG. 1B) was digested with EcoRI and subcloned into a plasmid vector. Plasmids that contained human DNA fragments were selected by colony hybridization using total human DNA as a probe. These clones were then used to search for cross-hybridizing sequences as described above for TB1, and the cross-hybridizing clones were subsequently used to screen cDNA libraries. One of the cDNA clones discovered in this way (FH38) contained a long ORF (2496 bp), 78 bp of which were identical to the above-noted sequences in YS11. The ends of the FH38 cDNA clone were then used to initiate cDNA walking to extend the sequence. Eventually, 85 cDNA clones were isolated from normal colon, brain and liver cDNA libraries and found to encompass 8973 nucleotides of contiguous transcript. The gene corresponding to this transcript was named APC. When used as probes for Northern blot analysis, APC cDNA clones hybridized to a single transcript of approximately 9.5 kb, suggesting that the great majority of the gene product was represented in the cDNA clones obtained. Sequences from the 5' end of the APC gene were found in YAC 37HG4 but not in YAC 14FH1. However, the 3' end of the APC gene was found in 14FH1 as well as 37HG4. Analogously, the 5' end of the MCC coding region was found in YAC clones 19AA9 and 266C3 but not 24ED6 or 14FH1, while the 3' end displayed the opposite pattern. Thus, MCC and APC transcription units pointed in opposite directions, with the direction of transcription going from centromeric to telomeric in the case of MCC, and telomeric to centromeric in the case of APC. PFGE analysis of YAC DNA digested with various restriction endonucleases showed that TB2 and SRP were between MCC and APC, and that the 3' ends of the coding regions of MCC and APC were separated by approximately 150 kb (FIG. 1B).

*2m ap8* [Sequence analysis of the APC cDNA clones revealed an open reading frame of 8,535 nucleotides. The 5' end of the ORF contained a methionine codon (codon 1) that was preceded by an in-frame stop codon 9 bp upstream, and the 3' end was followed by several in-frame stop codons. The protein produced by initiation at codon 1 would contain 2,842 amino acids (SEQ ID NO:7) (FIG. 3). The results of database searching with the APC gene product were quite complex due to the presence of large segments with locally biased amino acid compositions. In spite of this, APC could be roughly divided into two domains. The N-terminal 25% of the protein had a high content of leucine residues (12%) and showed local sequence similarities to myosins, various intermediate filament proteins (e.g., desmin, vimentin, neurofilaments) and *Drosophila* armadillo/human plakoglobin. The latter protein is a component of adhesive junctions (desmosomes) joining epithelial cells (Franke et al., Proc. Natl. Acad. Sci. U.S.A., Vol. 86, p. 4027 (1989); Perfer et al., Cell, Vol. 63, p. 1167 (1990)). The C-terminal 75% of APC (residues 731-2832) is 17% serine by composition with serine residues more or less uniformly distributed. This large domain also contains local concentrations of charged (mostly acidic) and proline residues. There was no indication of potential signal peptides, transmembrane regions, or nuclear targeting signals in APC, suggesting a cytoplasmic localization.]

To detect short similarities to APC, a database search was performed using the PAM-40 matrix (Altschul. J. Mol. Bio., Vol. 219, p. 555 (1991)). Potentially interesting matches to several proteins were found. The most suggestive of these

involved the *ral2* gene product of yeast, which is implicated in the regulation of *ras* activity (Fukui et al., *Mol. Cell. Biol.*, Vol. 9, p. 5617 (1989)). Little is known about how *ral2* might interact with *ras* but it is interesting to note the positively-charged character of this region in the context of the negatively-charged GAP interaction region of *ras*. A specific electrostatic interaction between *ras* and GAP-related proteins has been proposed.

*see 9.127*

[Because of the proximity of the MCC and APC genes, and the fact that both are implicated in colorectal tumorigenesis, we searched for similarities between the two predicted proteins. Bourne has previously noted that MCC has the potential to form alpha helical coiled coils (*Nature*, Vol. 351, p. 188 (1991)). Lupas and colleagues have recently developed a program for predicting coiled coil potential from primary sequence data (*Science*, Vol. 252, p. 1162 (1991)) and we have used their program to analyze both MCC and APC. Analysis of MCC indicated a discontinuous pattern of coiled-coil domains separated by putative "hinge" or "spacer" regions similar to those seen in laminin and other intermediate filament proteins. Analysis of the APC sequence revealed two regions in the N-terminal domain which had strong coiled coil-forming potential, and these regions corresponded to those that showed local similarities with myosin and IF proteins on database searching. In addition, one other putative coiled coil region was identified in the central region of APC. The potential for both APC and MCC to form coiled coils is interesting in that such structures often mediate homo- and hetero-oligomerization.]

Finally, it had previously been noted that MCC shared a short similarity with the region of the m3 muscarinic acetylcholine receptor (mAChR) known to regulate specificity of G-protein coupling. The APC gene also contained a local similarity to the region of the m3 mAChR (SEQ ID NO:9) that overlapped with the MCC similarity (SEQ ID NO:10) (FIG. 4B). Although the similarities to *ral2* (SEQ ID NO:8) (FIG. 4A) and m3 mAChR (SEQ ID NO:9) (FIG. 4B) were not statistically significant, they were intriguing in light of previous observations relating G-proteins to neoplasia.

Each of the six genes described above was expressed in normal colon mucosa, as indicated by their representation in colon cDNA libraries. To study expression of the genes in neoplastic colorectal epithelium, we employed reverse transcription-polymerase chain reaction (PCR) assays. Primers based on the sequences of FER, TB1, TB2, MCC, and APC were each used to design primers for PCR performed with cDNA templates. Each of these genes was found to be expressed in normal colon, in each of ten cell lines derived from colorectal cancers, and in tumor cell lines derived from lung and bladder tumors. The ten colorectal cancer cell lines included eight from patients with sporadic CRC and two from patients with FAP.

## EXAMPLE 2

This example demonstrates a genetic analysis of the role of the FER gene in FAP and sporadic colorectal cancers.

We considered FER as a candidate because of its proximity to the FAP locus as judged by physical and genetic criteria (see Example 1), and its homology to known tyrosine kinases with oncogenic potential. Primers were designed to PCR-amplify the complete coding sequence of FER from the RNA of two colorectal cancer cell lines derived from FAP patients. cDNA was generated from RNA and used as a template for PCR. The primers used were 5'-AGAAGGATCCCTTGTGCAGTGTGGA-3' (SEQ ID NO:95) and 5'-GACAGGATCCTGAAGCTGAGTTTG-3'

(SEQ ID NO:96). The underlined nucleotides were altered from the true FER sequence to create BamHI sites. The cell lines used were JW and Difi, both derived from colorectal cancers of FAP patients. (C. Paraskeva, B. G. Buckle, D. Sheer, C. B. Wigley, *Int. J. Cancer* 34, 49 (1984); M. E. Gross et al., *Cancer Res.* 51, 1452 (1991). The resultant 2554 basepair fragments were cloned and sequenced in their entirety. The PCR products were cloned in the BamHI site of Bluescript SK (Stratagene) and pools of at least 50 clones were sequenced en masse using T7 polymerase, as described in Nigro et al., *Nature* 342, 705 (1989).

Only a single conservative amino acid change (GTG→CTG, creating a val to leu substitution at codon 439) was observed. The region surrounding this codon was then amplified from the DNA of individuals without FAP and this substitution was found to be a common polymorphism, not specifically associated with FAP. Based on these results, we considered it unlikely (though still possible) the FER gene was responsible for FAP. To amplify the regions surrounding codon 439, the following primers were used: 5'-TCAGAAAGTGCTGAAGAG-3' (SEQ ID NO:97) and 5'-GGAATAATTAGGTCTCCAA-3' (SEQ ID NO:98). PCR products were digested with PstI, which yields a 50 bp fragment if codon 439 is leucine, but 26 and 24 bp fragments if it is valine. The primers used for sequencing were chosen from the FER cDNA sequence in Hao et al., *supra*.

### EXAMPLE 3

This example demonstrates the genetic analysis of MCC, TB2, SRP and APC in FAP and sporadic colorectal tumors. Each of these genes is linked and encompassed by contig 3 (see FIG. 1).

Several lines of evidence suggested that this contig was of particular interest. First, at least three of the four genes in this contig were within the deleted region identified in two FAP patients. (See Example 5 *infra*.) Second, allelic deletions of chromosome 5q21 in sporadic cancers appeared to be centered in this region. (Ashton-Rickardt et al., *Oncogene*, in press; and Miki et al., *Japn. J. Cancer Res.*, in press.) Some tumors exhibited loss of proximal RFLP markers (up to and potentially including the 5' end of MCC), but no loss of markers distal to MCC. Other tumors exhibited loss of markers distal to and perhaps including the 3' end of MCC, but no loss of sequences proximal to MCC. This suggested either that different ends of MCC were affected by loss in all such cases, or alternatively, that two genes (one proximal to and perhaps including MCC, the other distal to MCC) were separate targets of deletion. Third, clones from each of the six FAP region genes were used as probes on Southern blots containing tumor DNA from patients with Sporadic CRC. Only two examples of somatic changes were observed in over 200 tumors studied: a rearrangement/deletion whose centromeric end was located within the MCC gene (Kinzler et al., *supra*) and an 800 bp insertion within the APC gene between nucleotides 4424 and 5584. Fourth, point mutations of MCC were observed in two tumors (Kinzler et al.) *supra* strongly suggesting that MCC was a target of mutation in at least some sporadic colorectal cancers.

Based on these results, we attempted to search for subtle alterations of contig 3 genes in patients with FAP. We chose to examine MCC and APC, rather than TB2 or SRP, because of the somatic mutations in MCC and APC noted above. To facilitate the identification of subtle alterations, the genomic sequences of MCC and APC exons were determined (see Table I, SEQ ID NO:24-38).



TABLE I

## APC EXONS

EXON NUCLEOTIDES <sup>1</sup>	EXON BOUNDARY SEQUENCE <sup>2</sup>
822 to 930	<u>catgatgttatctgtatttaacctatagctcaattataccaatcctataatgcttaatttttag/GGTTCA</u> ... (SEQ ID NO: 24) ... ACCAAG/gtaacagaagattacaaacctggtcactaatgcatgactacttgcctaaag (SEQ ID NO: 25)
931 to 1309	<u>ggatattaaggtcgttaattttgttctaaactcattggccacag/GTGGAA</u> ... (SEQ ID NO: 26) ... ATCCAA/gtatgtctctataggtacatcgtatgcatg (SEQ ID NO: 27)
1310 to 1405	<u>catcattgctctcaatacaaaagcaataggtttatgtgattttattttcag/TGCCAG</u> ... (SEQ ID NO: 28) ... AACTAG/gtaagacaaaaatgtttttatgacatagacaactactggtg (SEQ ID NO: 29)
1406 to 1545	<u>tagatgatgtctttttctcttgcccttttaaatag/GGGGAC</u> ... (SEQ ID NO: 30) ... AACAAAG/gtatgtttataacatgtatttttaaggtatagctcaggtatga (SEQ ID NO: 31)
1546 to 1623	<u>gcttgctcaaggtgtcttttaataatgatcctctattctgtattttatctacag/GCTACG</u> ... (SEQ ID NO: 32) ... CAGCAG/gtactatitagaatttcacctgtttttctttttctttttttgaggcagggtctcacttg (SEQ ID NO: 33)
1624 to 1740	<u>gcaactagtatgattttatgtataaattatctaaatgatttaattgacag/GTTATT</u> ... (SEQ ID NO: 34) ... AAAAAAG/gtaaccttgaaaacatttagtactataatgaatttcattg (SEQ ID NO: 35)
1741 to 1955	<u>caactctaattagatgaccatatttcagaaacttactag/GAATCA</u> ... (SEQ ID NO: 36) ... CCACAG/gtatatatagattttatattcttttaaggtacagaattctactctcaaaaa (SEQ ID NO: 37)
1956 to 8973	<u>tcttgattttatttcag/GCAAAT</u> ... (SEQ ID NO: 38) ... GGTATTATGCAAAAAAATGTTTTTGT (SEQ ID NO: 1)

<sup>1</sup>Relative to predicted translation initiation site<sup>2</sup>Small case letters represent introns, large case letters represent exons

The entire 3' end of the cloned APC cDNA (nt 1956-8973) appeared to be encoded in this exon, as indicated by restriction endonuclease mapping and sequencing of the cloned genomic DNA. The ORF ended at nt 8535. The extreme 3' end of the APC transcript has not yet been identified.

These sequences were used to design primers for PCR analysis of constitutional DNA from FAP patients.

We first amplified eight exons and surrounding introns of the MCC gene in affected individuals from 90 different FAP kindreds. The PCR products were analyzed by a ribonuclease (RNase) protein assay. In brief, the PCR products were hybridized to in vitro transcribed RNA probes representing the normal genomic sequences. The hybrids were digested with RNase A, which can cleave at single base pair mismatches within DNA-RNA hybrids, and the cleavage products were visualized following denaturing gel electrophoresis. Two separate RNase protection analyses were performed for each exon, one with the sense and one with the antisense strand. Under these conditions, approximately 40% of all mismatches are detectable. Although some amino acid variants of MCC were observed in FAP patients, all such variants were found in a small percentage of normal individuals. These variants were thus unlikely to be responsible for the inheritance of FAP.

[We next examined three exons of the APC gene. The three exons examined included those containing nt 822-930, 931-1309, and the first 300 nt of the most distal exon (nt 1956-2256). PCR and RNase protection analysis were performed as described in Kinzler et al. supra, using the primers underlined in Table I (SEQ ID NO:24-38). The primers for nt 1956-2256 were 5'-GCAAATCCTAAGAGAGAACAA-3' (SEQ ID NO:99) and 5'-GATGGCAAGCTTGAGCCAG-3' (SEQ ID NO:100).]

[In 90 kindreds, the RNase protection method was used to screen for mutations and in an additional 13 kindreds, the PCR products were cloned and sequenced to search for mutations not detectable by RNase protection. PCR products were cloned into a Bluescript vector modified as described in T. A. Holton and M. W. Graham, Nucleic Acids Res. 19, 1156 (1991). A minimum of 100 clones were pooled and sequenced. Five variants were detected among the 103 kindreds analyzed. Cloning and subsequent DNA sequencing of the PCR product of patient P21 indicated a C to T transition in codon 413 that resulted in a change from arginine to cysteine. This amino acid variant was not observed in any of 200 DNA samples from individuals without FAP. Cloning and sequencing of the PCR product

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from patients P24 and P34, who demonstrated the same abnormal RNase protection pattern indicated that both had a C to T transition at codon 801 that resulted in a change from arginine (CGA) to a stop codon (TGA). This change was not present in 200 individuals without FAP. As this point mutation resulted in the predicted loss of the recognition site for the enzyme Taq I, appropriate PCR products could be digested with Taq I to detect the mutation. This allowed us to determine that the stop codon co-segregated with disease phenotype in members of the family of P24. The inheritance of this change in affected members of the pedigree provides additional evidence for the importance of the mutation.]

Cloning and sequencing of the PCR product from FAP patient P93 indicated a C to G transversion at codon 279, also resulting in a stop codon (change from TCA to TGA). This mutation was not present in 200 individuals without FAP. Finally, one additional mutation resulting in a serine (TCA) to stop codon (TGA) at codon 712 was detected in a single patient with FAP (patient P60).

The five germline mutations identified are summarized in Table IIA, as well as four others discussed in Example 9.

TABLE IIA

Germline mutations of the APC gene in FAP and GS Patients

EXTRA-COLONIC PATIENT DISEASE	CODON	NUCLEOTIDE CHANGE	AMINO ACID CHANGE	AGE	
93	279	TCA->TGA	Ser->Stop	39	Mandibular
<u>Osteoma</u>					
24	301	CGA->TGA	Arg->Stop	46	None
34	301	CGA->TGA	Arg->Stop	27	Desmoid
<u>Tumor</u>					
65 21	413	CGC->TGC	Arg->Cys	24	Mandibular

TABLE IIA-continued

Germline mutations of the APC gene in FAP and GS Patients					5
EXTRA-COLO-NIC PATIENT DISEASE	CODON	NUCLEO-TIDE CHANGE	AMINO ACID CHANGE	AGE	
<u>Osteoma</u>					10
60	712	TCA-> <u>TGA</u>	Ser->Stop	37 Mandi-bular	
<u>Osteoma</u>					15
3746	243	CAGAG->CAG	splice-junction		
3460	301	CGA-> <u>TGA</u>	Arg->Stop		
3827	456	CTTCA->CTTCA	frameshift		
3712	500	T-> <u>G</u>	Tyr->Stop		
* The mutated nucleotides are underlined.					20

Jan. 2.5 [In addition to these germline mutations, we identified several somatic mutations of MCC and APC in sporadic CRC's. Seventeen MCC exons were examined in 90 sporadic colorectal cancers by RNase protection analysis. In each case where an abnormal RNase protection pattern was observed, the corresponding PCR products were cloned and sequenced. This led to the identification of six point mutations (two described previously) (Kinzler et al., supra), each of which was not found in the germline of these patients (Table IIB).]

TABLE IIB

Somatic Mutations in Sporadic CRC Patients				35
PATIENT	CODON <sup>1</sup>	NUCLEOTIDE CHANGE	AMINO ACID CHANGE	
T35	MCC 12	GAG/ <u>gt</u> aaga->GAG/ <u>gt</u> aaaa	(Splice Donor)	
T16	MCC 145	ctcag/ <u>GGA</u> ->gtcag/ <u>GGA</u>	(Splice Acceptor)	40
T47	MCC 267	CGG->CTG	Arg->Leu	
T81	MCC 490	TCG->TTG	Ser->Leu	
T35	MCC 506	CGG->CAG	Arg->Gln	
T91	MCC 698	GCT->GTT	Ala->Val	
T34	APC 288	CCAGT->CC <u>AGCC</u> AGT	(Insertion)	45
T27	APC 331	CGA->TGA	Arg->Stop	
T135	APC 437	CAA/ <u>g</u> taa->CAA/ <u>g</u> caa	(Splice Donor)	
T20I	APC 1338	CAG-> <u>TAG</u>	Gln->Stop	

For splice site mutations, the codon nearest to the mutation is listed. The underlined nucleotides were mutant; small case letters represent introns, large case letters represent exons. 50

Four of the mutations resulted in amino acid substitutions and two resulted in the alteration of splice site consensus elements. Mutations at analogous splice site positions in other genes have been shown to alter RNA processing in vivo and in vitro. 55

Three exons of APC were also evaluated in sporadic tumors. Sixty tumors were screened by RNase protection, and an additional 98 tumors were evaluated by sequencing. The exons examined included nt 822-930, 931-1309, and 1406-1545 (Table I). A total of three mutations were identified, each of which proved to be somatic. Tumor T27 contained a somatic mutation of CGA (arginine) to TGA (stop codon) at codon 33. Tumor T135 contained a GT to GC change at a splice donor site. Tumor T34 contained a 5 bp insertion (CAGCC between codons 288 and 289) resulting in a stop at codon 291 due to a frameshift. 60 65

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We serendipitously discovered one additional somatic mutation in a colorectal cancer. During our attempt to define the sequences and splice patterns of the MCC and APC gene products in colorectal epithelial cells, we cloned cDNA from the colorectal cancer cell line SW480. The amino acid sequence of the MCC gene from SW480 was identical to that previously found in clones from human brain. The sequence of APC in SW480 cells, however, differed significantly, in that a transition at codon 1338 resulted in a change from glutamine (CAG) to a stop codon (TAG). To determine if this mutation was somatic, we recovered DNA from archival paraffin blocks of the original surgical specimen (T201) from which the tumor cell line was derived 28 years ago.

DNA was purified from paraffin sections as described in S. E. Goelz, S. R. Hamilton, and B. Vogelstein. *Biochem. Biophys. Res. Comm.* 130, 118 (1985). PCR was performed as described in reference 24, using the primers 5'-GTTCCAGCAGTGTACAG-3' (SEQ ID NO:101) and 5'-GGGAGATTTCGCTCCTGA-3' (SEQ ID NO:102). A PCR product containing codon 1338 was amplified from the archival DNA and used to show that the stop codon represented a somatic mutation present in the original primary tumor and in cell lines derived from the primary and metastatic tumor sites, but not from normal tissue of the patient.

The ten point mutations in the MCC and APC genes so far discovered in sporadic CRCs are summarized in Table IIB. Analysis of the number of mutant and wild-type PCR clones obtained from each of these tumors showed that in eight of the ten cases, the wild-type sequence was present in approximately equal proportions to the mutant. This was confirmed by RFLP analysis using flanking markers from chromosome 5q which demonstrated that only two of the ten tumors (T135 and T201) exhibited an allelic deletion on chromosome 5q. These results are consistent with previous observations showing that 20-40% of sporadic colorectal tumors had allelic deletions of chromosome 5q. Moreover, these data suggest that mutations of 5q21 genes are not limited to those colorectal tumors which contain allelic deletions of this chromosome.

#### EXAMPLE 4

This example characterizes small, nested deletions in DNA from two unrelated FAP patients.

DNA from 40 FAP patients was screened with cosmids that has been mapped into a region near the APC locus to identify small deletions or rearrangements. Two of these cosmids, L5.71 and L5.79, hybridized with a 1200 kb *NotI* fragment in DNAs from most of the FAP patients screened.

The DNA of one FAP patient, 3214, showed only a 940 kb *NotI* fragment instead of the expected 1200 kb fragment. DNA was analyzed from four other members of the patient's immediate family; the 940 kb fragment was present in her affected mother (4711), but not in the other, unaffected family members. The mother also carried a normal 1200 kb *NotI* fragment that was transmitted to her two unaffected offspring. These observations indicated that the mutant polyposis allele is on the same chromosome as the 940 kb *NotI* fragment. A simple interpretation is that APC patients 3214 and 4711 each carry a 260 kb deletion within the APC locus.

If a deletion were present, then other enzymes might also be expected to produce fragments with altered mobilities. Hybridization of L5.79 to *NruI*-digested DNAs from both affected members of the family revealed a novel *NruI*

fragment of 1300 kb, in addition to the normal 1200 kb NruI fragment. Furthermore, MluI fragments in patients 3214 and 4711 also showed an increase in size consistent with the deletion of an MluI site. The two chromosome 5 homologs of patient 3214 were segregated in somatic cell hybrid lines; HHW1155 (deletion hybrid) carried the abnormal homolog and HHW1159 (normal hybrid) carried the normal homolog.

*In 916* [Because patient 8214 showed a 940 kb NotI fragment, she had not inherited the 1200 kb fragment present in the unaffected father's DNA. This observation suggests that he must be heterozygous for, and have transmitted, either a deletion of the L5.79 probe region or a variant NotI fragment too large to resolve on the gel system. As expected, the hybrid cell line HHW1159, which carries the paternal homolog, revealed no resolved Not fragment when probed with L5.79. However, probing of HHW1159 DNA with L5.79 following digestion with other enzymes did reveal restriction fragments, demonstrating the presence of DNA homologous to the probe. The father is, therefore, interpreted as heterozygous for a polymorphism at the NotI site, with one chromosome 5 having a 1200 kb NotI fragment and the other having a fragment too large to resolve consistently on the gel. The latter was transmitted to patient 3214.]

*In 917* [When double digests were used to order restriction sites within the 1200 kb NotI fragment, L5.71 and L5.79 were both found to lie on a 550 kb NotI-NruI fragment and, therefore, on the same side of an NruI site in the 1200 kb NotI fragment. To obtain genomic representation of sequences present over the entire 1200 kb NotI fragment, we constructed a library of small-fragment inserts enriched for sequences from this fragment. DNA from the somatic cell hybrid HHW141, which contains about 40% of chromosome 5, was digested with NotI and electrophoresed under pulsed-field gel (PFGE) conditions; EcoRI fragments from the 1200 kb region of this gel were cloned into a phage vector. Probe Map30 was isolated from this library. In normal individuals probe Map30 hybridizes to the 1200 kb NotI fragment and to a 200 kb NruI fragment. This latter hybridization places Map30 distal, with respect to the locations of L5.71 and L5.79, to the NruI site of the 550 kb NotI-NruI fragment.]

Because Map30 hybridized to the abnormal, 1300 kb NruI fragment of patient 3214, the locus defined by Map30 lies outside the hypothesized deletion. Furthermore, in normal chromosomes Map30 identified a 200 kb NruI fragment and L5.79 identified a 1200 kb NruI fragment; the hypothesized deletion must, therefore, be removing an NruI site, or sites, lying between Map30 and L5.79, and these two probes must flank the hypothesized deletion. A restriction map of the genomic region, showing placement of these probes, is shown in FIG. 5.

A NotI digest of DNA from another FAP patient, 3824, was probed with L5.79. In addition to the 1200 kb normal NotI fragment, a fragment of approximately 1100 kb was observed, consistent with the presence of a 100 kb deletion in one chromosome 5. In this case, however, digestion with NruI and MluI did not reveal abnormal bands, indicating that if a deletion were present, its boundaries must lie distal to the NruI and MluI sites of the fragments identified by L5.79. Consistent with this expectation, hybridization of Map30 to DNA from patient 3824 identified a 760 kb MluI fragment in addition to the expected 860 kb fragment, supporting the interpretation of a 100 kb deletion in this patient. The two chromosome 5 homologs of patient 3824 were segregated in somatic cell hybrid lines; HHW1291 was found to carry only the abnormal homolog and HHW1290 only the normal homolog.

That the 860 kb MluI fragment identified by Map30 is distinct from the 830 kb MluI fragment identified previously

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by L5.79 was demonstrated by hybridization of Map30 and L5.79 to a NotI-MluI double digest of DNA from the hybrid cell (HHW1159) containing the nondeleted chromosome 5 homolog of patient 3214. As previously indicated, this hybrid is interpreted as missing one of the NotI sites that define the 1200 kb fragment. A 620 kb NotI-MluI fragment was seen with probe L5.79, and an 860 kb fragment was seen with Map30. Therefore, the 830 kb MluI fragment recognized by probe L5.79 must contain a NotI site in HHW1159 DNA; because the 860 kb MluI fragment remains intact, it does not carry this NotI site and must be distinct from the 830 kb MluI fragment.

#### EXAMPLE 5

This example demonstrates the isolation of human sequences which span the region deleted in the two unrelated FAP patients characterized in Example 4.

A strong prediction of the hypothesis that patients 8214 and 3824 carry deletions is that some sequences present on normal chromosome 5 homologs would be missing from the hypothesized deletion homologs. Therefore, to develop genomic probes that might confirm the deletions, as well as to identify genes from the region, YAC clones from a contig seeded by cosmid L5.79 were localized from a library containing seven haploid human genome equivalents (Albertsen et al., Proc. Natl. Acad. Sci. U.S.A., Vol. 87, pp. 4256-4260 (1990)) with respect to the hypothesized deletions. Three clones, YACs 57B8, 310D8, and 183H12, were found to overlap the deleted region.

Importantly, one end of YAC 57B8 (clone AT57) was found to lie within the patient 3214 deletion. Inverse polymerase chain reaction (PCR) defined the end sequences of the insert of YAC 57B8. PCR primers based on one of these end sequences repeatedly failed to amplify DNA from the somatic cell hybrid (HHW1155) carrying the deleted homolog of patient 3214, but did amplify a product of the expected size from the somatic cell hybrid (HHW1159) carrying the normal chromosome 5 homolog. This result supported the interpretation that the abnormal restriction fragments found in the DNA of patient 3214 result from a deletion.

Additional support for the hypothesis of deletion in DNA from patient 3214 came from subcloned fragments of YAC 183H12, which spans the region in question. Y11, an EcoRI fragment cloned from YAC 183H12, hybridized to the normal, 1200 kb NotI fragment of patient 4711, but failed to hybridize to the abnormal, 940 kb NotI fragment of 4711 or to DNA from deletion cell line HHW1155. This result confirmed the deletion in patient 3214.

Two additional EcoRI fragments from YAC 183H12, Y10 and Y14, were localized within the patient 3214 deletion by their failure to hybridize to DNA from HHW1155. Probe Y10 hybridizes to a 150 kb NruI fragment in normal chromosome 5 homologs. Because the 3214 deletion creates the 1300 kb NruI fragment seen with the probes L5.79 and Map30 that flank the deletion, these NruI sites and the 150 kb NruI fragment lying between must be deleted in patient 3214. Furthermore, probe Y10 hybridizes to the same 620 kb NotI-MluI fragment seen with probe L5.79 in normal DNA, indicating its location as L5.79-proximal to the deleted MluI site and placing it between the MluI site and the L5.79-proximal NruI site. The MluI site must, therefore, lie between the NruI sites that define the 150 kb NruI fragment (see FIG. 5).

Probe Y11 also hybridized to the 150 kb NruI fragment in the normal chromosome 5 homolog, but failed to hybridize

to the 620 kb NotI-MluI fragment, placing it L5.79-distal to the MluI site, but proximal to the second NruI site. Hybridization to the same (860 kb) MluI fragment as Map30 confirmed the localization of probe Y11 L5.79-distal to the MluI site.

Probe Y14 was shown to be L5.79-distal to both deleted NruI sites by virtue of its hybridization to the same 200 kb NruI fragment of the normal chromosome 5 seen with Map30. Therefore, the order of these EcoRI fragments derived from YAC 183H12 and deleted in patient 3214, with respect to L5.79 and Map30, is L5.79-Y10-Y11-Y14-Map30.

The 100 kb deletion of patient 3824 was confirmed by the failure of aberrant restriction fragments in this DNA to hybridize with probe Y11, combined with positive hybridizations to probes Y10 and/or Y14. Y10 and Y14 each hybridized to the 1100 kb NotI fragment of patient 3824 as well as to the normal 1200 kb NotI fragment, but Y11 hybridized to the 1200 kb fragment only. In the MluI digest, probe Y14 hybridized to the 860 kb and 760 kb fragments of patient 3824 DNA, but probe Y11 hybridized only to the 860 kb fragment. We conclude that the basis for the alteration in fragment size in DNA from patient 3824 is, indeed, a deletion. Furthermore, because probes Y10 and Y14 are missing from the deleted 3214 chromosome, but present on the deleted 3824 chromosome, and they have been shown to flank probe Y11, the deletion in patient 3824 must be nested within the patient 3214 deletion.

Probes Y10, Y11, Y14 and Map30 each hybridized to YAC 310D8, indicating that this YAC spanned the patient 3824 deletion and at a minimum, most of the 3214 deletion. The YAC characterizations, therefore, confirmed the presence of deletions in the patients and provided physical representation of the deleted region.

#### EXAMPLE 6

This example demonstrates that the MCC coding sequence maps outside of the region deleted in the two FAP patients characterized in Example 4.

An intriguing FAP candidate gene, MCC, recently was ascertained with cosmid L5.71 and was shown to have undergone mutation in colon carcinomas (Kinzler et al., supra). It was therefore of interest to map this gene with respect to the deletions in APC patients. Hybridization of MCC probes with an overlapping series of YAC clones extending in either direction from L5.71 showed that the 3' end of MCC must be oriented toward the region of the two APC deletions.

Therefore, two 3' cDNA clones from MCC were mapped with respect to the deletions: clone 1CI (bp 2378-4181) and clone 7 (bp 2890-3560). Clone 1CI contains sequences from the C-terminal end of the open reading frame, which stops at nucleotide 2708, as well as 3' untranslated sequence. Clone 7 contains sequence that is entirely 3' to the open reading frame. Importantly, the entire 3' untranslated sequence contained in the cDNA clones consists of a single 2.5 kb exon. These two clones were hybridized to DNAs from the YACs spanning the FAP region. Clone 7 fails to hybridize to YAC 310D8, although it does hybridize to YACs 183H12 and 57B8; the same result was obtained with the cDNA 1CI. Furthermore, these probes did show hybridization to DNAs from both hybrid cell lines (HWW1159 and HWW1155) and the lymphoblastoid cell line from patient 3214, confirming their locations outside the deleted region. Additional mapping experiments suggested that the 3' end of the MCC cDNA clone contig is likely to be located more

than 45 kb from the deletion of patient 3214 and, therefore, more than 100 kb from the deletion of patient 3824.

## EXAMPLE 7

5 This example identifies three genes within the deleted region of chromosome 5 in the two unrelated FAP patients characterized in Example 4.

Genomic clones were used to screen cDNA libraries in three separate experiments. One screening was done with a phage clone derived from YAC 310D8 known to span the 260 kb deletion of patient 3214. A large-insert phage library was constructed from this YAC; screening with Y11 identified  $\lambda$ 205, which mapped within both deletions. When clone  $\lambda$ 205 was used to probe a random-, plus oligo(dT)-, primed fetal brain cDNA library (approximately 300,000 phage), six cDNA clones were isolated and each of them mapped entirely within both deletions. Sequence analysis of these six clones formed a single cDNA contig, but did not reveal an extended open reading frame. One of the six cDNAs was used to isolate more cDNA clones, some of which crossed the L5.71-proximal breakpoint of the 3824 deletion, as indicated by hybridization to both chromosome of this patient. These clones also contained an open reading frame, indicating a transcriptional orientation proximal to distal with respect to L5.71. This gene was named DP1 (deleted in polyposis 1). This gene is identical to TB2 described above.

cDNA walks yielded a cDNA contig of 3.0-3.5 kb, and included two clones containing terminal poly(A) sequences. This size corresponds to the 3.5 kb band seen by Northern analysis. Sequencing of the first 3163 bp of the cDNA contig revealed an open reading frame extending from the first base to nucleotide 631, followed by a 2.5 kb 3' untranslated region. The sequence surrounding the methionine codon at base 77 conforms to the Kozak consensus of an initiation methionine (Kozak, 1984). Failed attempts to walk farther, coupled with the similarity of the lengths of isolated cDNA and mRNA, suggested that the NH<sub>2</sub>-terminus of the DP1 protein had been reached. Hybridization to a combination of genomic and YAC DNAs cut with various enzymes indicated the genomic coverage of DP1 to be approximately 30 kb.

*See 913* [Two additional probes for the locus, YS-11 and YS-39, which had been ascertained by screening of a cDNA library with an independent YAC probe identified with MCC sequences adjacent to L5.71, were mapped into the deletion region. YS-39 was shown to be a cDNA identical in sequence to DP1. Partial characterization of YS-11 had shown that 200 bp of DNA sequence at one end was identical to sequence coding for the 19 kd protein of the ribosomal signal recognition particle, SRP19 (Lingelbach et al., supra). Hybridization experiments mapped YS-11 within both deletions. The sequence of this clone, however, was found to be complex. Although 454 bp of the 1032 bp sequence of YS-11 were identical to the GenBank entry for the SRP19 gene, another 578 bp appended 5' to the SRP19 sequence was found to consist of previously unreported sequence containing no extended open reading frames. This suggested that YS-11 was either a chimeric clone containing two independent inserts or a clone of an incompletely processed or aberrant message. If YS-11 were a conventional chimeric clone, the independent segments would not be expected to map to the same physical region. The segments resulting from anomalous processing of a continuous transcript, however, would map to a single chromosomal region.]



Inverse PCR with primers specific to the two ends of YS-11, the SRP19 .end and the unidentified region, verified that both sequences map within the YAC 310D8; therefore, YS-11 is most likely a clone of an immature or anomalous mRNA species. Subsequently, both ends were shown to lie with the deleted region of patient 3824, and YS-11 was used to screen for additional cDNA clones.

Of the 14 cDNA clones selected from the fetal brain library, one clone, V5, was of particular interest in that it contained an open reading frame throughout, although it included only a short identity to the first 78 5' bases of the YS-11 sequence. Following the 78 bp of identical sequence, the two cDNA sequences diverged at an AG. Furthermore, divergence from genomic sequence was also seen after these 78 bp, suggesting the presence of a splice junction, and supporting the view that YS-11 represents an irregular message.

Starting with V5, successive 5' and 3' walks were performed; the resulting cDNA contig consisted of more than 100 clones, which defined a new transcript, DP2. Clones walking in the 5' direction crossed the 3824 deletion breakpoint farthest from L5.71; since its 3' end is closer to this cosmid than its 5' end, the transcriptional orientation of DP2 is opposite to that of MCC and DP1.

The third screening approach relied on hybridization with a 120 kb MluI fragment from YAC 57B8. This fragment hybridizes with probe Y11 and completely spans the 100 kb deletion in patient 3824. the fragment was purified on two preparative PFGs, labeled, and used to screen a fetal brain cDNA library. A number of cDNA clones previously identified in the development of the DP1 and DP2 contigs were reascertained. However, 19 new cDNA clones mapped into the patient 3824 deletion. Analysis indicated that these 19 formed a new contig, DP3, containing a large open reading frame.

A clone from the 5' end of this new cDNA contig hybridized to the same EcoRI fragment as the 3' end of DP2. Subsequently, the DP2 and DP3 contigs were connected by a single 5' walking step from DP3, to form the single contig DP2.5. The complete nucleotide sequence of DP2.5 is shown in FIG. 9.

The consensus cDNA sequence of DP2.5 suggests that the entire coding sequence of DP2.5 has been obtained and is 8532 bp long. The most 5' ATG codon occurs two codons from an in-frame stop and conforms to the Kozak initiation consensus (Kozak, Nucl. Acids. Res., Vol. 12, p. 857-872 1984). The 3' open reading frame breaks down over the final 1.8 kb, giving multiple stops in all frames. A poly(A) sequence was found in one clone approximately 1 kb into the 3' untranslated region, associated with a polyadenylation signal 33 bp upstream (position 9530). The open reading frame is almost identical to that identified as APC above.

An alternatively spliced exon at nucleotide 934 of the DP2.5 transcript is of potential interest. it was first discovered by noting that two classes of cDNA had been isolated. The more abundant cDNA class contains a 303 bp exon not included in the other. The presence in vivo of the two transcripts was verified by an exon connection experiment. Primers flanking the alternatively spliced exon were used to amplify, by PCR, cDNA prepared from various adult tissues. Two PCR products that differed in size by approximately 300 bases were amplified from all the tissues tested; the larger product was always more abundant than the smaller.

## EXAMPLE 8

This example demonstrates the primers used to identify subtle mutations in DP1, SRP19, and DP25.

To obtain DNA sequence adjacent to the exons of the genes DP1, DP2.5, and SRP19, sequencing substrate was obtained by inverse PCR amplification of DNAs from two YACs, 310D8 and 183H12, that span the deletions. Ligation at low concentration cyclized the restriction enzyme-digested YAC DNAs. Oligonucleotides with sequencing tails, designed in inverse orientation at intervals along the cDNAs, primed PCR amplification from the cyclized templates. Comparison of these DNA sequences with the cDNA sequences placed exon boundaries at the divergence points. SRP19 and DP1 were each shown to have five exons. DP2.5 consisted of 15 exons. The sequences of the oligonucleotides synthesized to provide PCR amplification primers for the exons of each of these genes are listed in Table III SEQ ID NO:39-94.

TABLE III

## Sequences of Primers Used for SSCP Analyses

Exon	Primer 1	Primer 2
<u>DP1</u>		
	UP-TCCCGCGCTGCGCTCTC	RP-GCAGCGGCGGCTCCCGTG
	UP-GTGAACGGCTCTCATGCTGC	RP-AOGTGCGGGGAGGAATGGA
	UP-ATGATATCTTACCAAATGATATAC	RP-TTATTCCTACTTCTCTATACAG
	UP-TACCCATGCTGGCTCTTTTC	RP-TGGGGCCATCTTGTTCCTGA
	UP-ACATTAGGCACAAAGCTTGCAA	RP-ATCAAGCTCCAGTAAGAAGGTA
<u>SRP19</u>		
	UP-TGCGGCTCTGGGTGTGTG	RP-GCCCTTCTTCTGAGGAC
	UP-TTTTCTCTGCTCTTACTGC	RP-ATGACACCCOCCATTCCTC
	UP-CCACTTAAAGCACATATATTAGT	RP-GTATGGAAAATAGTGAAGAACC
	UP-TTCTTAAAGTCCTGTTTCTTTTG	RP-TTTAGAACCCTTTTGTGTGTG
	UP-CTCAGATTATACACTAAGCCTAAC	RP-CATGTCTCTTACAGTAGTACCA
<u>DP25</u>		
	UP-AGGTCCAAGGGTAGCCAAGG*	RP-TAAAAATGGATAAACTACAATTAAAAG
	UP-AAATACAGAATCATGTCTTGAAGT	RP-ACACCTAAAGATGACAATTGAG
	UP-TAACTTAGATAGCAGTAATTCCC*	RP-ACAATAAACTGGAGTACACAAGG
	UP-ATAGGTCATTGCTTCTTGCTGAT*	RP-TGAATTTTAATGGATTACCTAGGT
	UP-CTTTTTTTGCTTTTACTGATTAAAG	RP-TGTAATTCATTTTATTCCTAATACCTC
	UP-GGTAGCCATAGTATGATTATTCT	RP-CTACCTATTTTATACCCACAAC

TABLE III-continued

Sequences of Primers Used for SSCP Analyses		
Exon	Primer 1	Primer 2
	UP-AAGAAAGCCTACACCATTTTTCG	RP-GATCATTCTTAGAACCATCTTGC
	UP-ACCTATAGTCTAAATTATACCATC	RP-GTCATGGCATTACTGACCAG
	UP-AGTGTGTAATTTTGTCTTAACTC	RP-TGAAGGACTCCGATTTTCAACC*
	UP-TCATTCACTCACAGCCTGATGAC*	RP-GCTTTTGAAACATGCATACGAT
	UP-AAACATCATTCCTCTTCAAATAAC	RP-TACCATGATTTTAAATAATCCACCAG
	UP-GATGATTGCTCTTTTCTCTTTG	RP-CTGAGCTATCTTAAGAAATACATG
	UP-TTTTAAATGATCCTCTTATCTGTAT	RP-ACAGAGTCAGACCCCTCCCTCAAAG
	UP-TTCTTATCTTACTGCTAGCAAT	RP-ATACACAGGTAAGAAATTAGGA
	UP-TAGATGACCCATATCTCTTTC	RP-CAATTAGGCTCTTTTGAGAGTA
3-A	UP-GTTACTGCATACACATTGTGAC	RP-GCTTTTGTGTTTGTAAACATGAAG*
-B	UP-AGTACAAGGATGCCAATATTATG*	RP-ACCTTCTATCTTTTTCAGAACGAG*
-C	UP-ATTGTAATACTACAGTGTACCC*	RP-CTTGTATCTTAATTGGCATAAGG*
-D	UP-CTGCCCATACACATTCAAACAC*	RP-TGTTTGGCTCTTGGCCATCTT*
-E	UP-AGTCTTAAATATTTCAGATGAGCAG*	RP-GTTTCTCTTCTTATATTTTATGCTA*
-F	UP-AAGCCTACCAATTATAGTGAACG*	RP-AGCTGATGACAAAGATGATAATC*
-G	UP-AAGAAACAATACAGACTTATTGTG*	RP-ATGAGTGGGGTCTCTCTGAAC*
-H	UPATCTCCCTCCAAAGTGGTGC*	RP-TCCATCTGGAGTACTTCTGTG*
-I	UP-AGTAAATGCTGTCAGTTCAGAGG*	RP-CGGTGGCATATCATCCCC*
-J	UP-CCCAGACTGCTTCAAATTACC*	RP-GAGCCTCATCTGTACTTCTGC*
-K	UP-CCCTCCAAATGAGTTAGCTGC*	RP-TTGTGGTATAGGTTTACTGGTG*
-L	UP-ACCCAACAAAATCAGTTAGATG*	RP-GTGGCTGTAACTTTAGCCTC*
-N	UP-ATGATGTTGACCTTCCAGGG*	RP-ATTGTGTAACCTTTTCATCAGTTGC*
-M	UP-AAAGACATACCAGACAGAGGG*	RP-CTTTTTTGGCATTGGGGAGCT*
-O	UP-AAGATGACCTGTGTCAGGAATG*	RP-GAATCAGACCAAGCTTGTCTAGAT*
-P	UP-CAATAGTAAGTAGTTTACATCAAG*	RP-AAACAGGACTTGTACTGTAGGA*
-Q	UP-CAGCCCTTCAAGCAAACATC*	RP-GAGGACTTATTCATTTCTACC*
-R	UP-CAGTCTCCTGGCCGAAACATC*	RP-GTTGACTGGCGTACTAATACAG*
-S	UP-TGGTAATGGAGCCAATAAAAAGG*	RP-TGGGACTTTTGGCCATCCAC*
-T	UP-TGTCTCTATCCACATTCGTC*	RP-ATGTTTTTTCATCTCCTCTTTTGC*
-U	UP-GGAGAAGAACTGGAAGTTTCATC*	RP-TTGAAICTTAAATGTTTGGATTTCG*
-V	UP-TCTCCACAGGTAATACTCC	RP-GCTACAACCTGAATGGGTACG
-W	UP-CAGGACAAAATAATCCTGTCC	RP-ATTTCTTACTTTTCATCTCTCTC

All primers are read in the 5' to 3' direction, the first primer in each pair lies 5' of the exon it amplifies; the second primer lies 3' of the exon it amplifies. Primers that lie within the exon are identified by an asterisk. UP represents the -21M13 universal primer sequence; RP represents the M13 reverse primer sequence.

With the exception of exons 1, 3, 4, 9, and 15 of DP2.5 (see below), the primer sequences were located in intron sequences flanking the exons. The 5' primer of exon 1 is complementary to the cDNA sequence, but extends just into the 5' Kozak consensus sequence for the initiator methionine, allowing a survey of the translated sequences. The 5' primer of exon 3 is actually in the 5' coding sequences of this exon, as three separate intronic primers simply would not amplify. The 5' primer of exon 4 just overlaps the 5' end of this exon, and we thus fail to survey the 19 most 5' bases of this exon. For exon 9, two overlapping primer sets were used, such that each had one end within the exon. For exon 15, the large 3' exon of DP2.5, overlapping primer pairs were placed along the length of the exon; each pair amplified a product of 250-400 bases.

#### EXAMPLE 9

This example demonstrates the use of single stranded conformation polymorphism (SSCP) analysis as described by Orita et al. Proc. Natl. Acad. Sci. U.S.A., Vol. 86, pp. 2766-70 (1989) and Genomics, Vol. 5, pp. 874-879 (1989) as applied to DP1, SRP19 and DP2.5.

SSCP analysis identifies most single- or multiple-base changes in DNA fragments up to 400 bases in length. Sequence alterations are detected as shifts in electrophoretic mobility of single-stranded DNA on nondenaturing acrylamide gels; the two complementary strands of a DNA segment usually resolve as two SSCP conformers of distinct mobilities. However, if the sample is from an individual heterozygous for a base-pair variant within the amplified

segment, often three or more bands are seen. In some cases, even the sample from a homozygous individual will show multiple bands. Base-pair-change variants are identified by differences in pattern among the DNAs of the sample set.

Exons of the candidate genes were amplified by PCR from the DNAs of 61 unrelated FAP patients and a control set of 12 normal individuals. The five exons from DP1 revealed no unique conformers in the FAP patients, although common conformers were observed with exons 2 and 3 in some individuals of both affected and control sets, indicating the presence of DNA sequence polymorphisms. Likewise, none of the five exons of SRP19 revealed unique conformers in DNA from FAP patients in the test panel.

Testing of exons 1 through 14 and primer sets A through N of exon 15, of the DP2.5 gene, however, revealed variant conformers specific to FAP patients in exons 7, 8, 10, 11, and 15. These variants were in the unrelated patients 3746, 3460, 3827, 3712, and 3751, respectively. The PCR-SSCP procedure was repeated for each of these exons in the five affected individuals and in an expanded set of 48 normal controls. The variant bands were reproducible in the FAP patients but were not observed in any of the control DNA samples. Additional variant conformers in exons 11 and 15 of the DP2.5 gene were seen; however, each of these was found in both the affected and control DNA sets. The five sets of conformers unique to the FAP patients were sequenced to determine the nucleotide changes responsible for their altered mobilities. The normal conformers from the host individuals were sequenced also. Bands were cut from the dried acrylamide gels, and the DNA was eluted. PCR amplification of these DNAs provided template for sequencing.

*In. AP12* The sequences of the unique conformers from exons 7, 8, 10, and 11 of DP2.5 revealed dramatic mutations in the DP2.5 gene. The sequence of the new mutation creating the exon 7 conformer in patient 3746 was shown to contain a deletion of two adjacent nucleotides, at positions 730 and 731 in the cDNA sequence (FIG. 7, SEQ ID NO:1). The normal sequence at this splice junction is CAGGGTCA (intronic sequence underlined), with the intron-exon boundary between the two repetitions of AG. The mutant allele in this patient has the sequence CAGGTCA. Although this change is at the 5' splice site, comparison with known consensus sequences of splice junctions would suggest that a functional splice junction is maintained. If this new splice junction were functional, the mutation would introduce a frameshift that creates a stop codon 15 nucleotides downstream. If the new splice junction were not functional, messenger processing would be significantly altered.]

To confirm the 2-base deletion, the PCR product from FAP patient 3746 and a control DNA were electrophoresed on an acrylamide-urea denaturing gel, along with the products of a sequencing reaction. The sample from patient 3746 showed two bands differing in size by 2 nucleotides, with the larger band identical in mobility to the control sample; this result was independent confirmation that patient, 3746 is heterozygous for a 2 bp deletion.

*229717* The unique conformer found in exon 8 of patient 3460 was found to carry a C-T transition, at position 904 in the cDNA sequence of DP2.5 (shown in FIG. 7), which replaced the normal sequence of CGA with TGA. This point mutation, when read in frame, results in a stop codon replacing the normal arginine codon. This single-base change had occurred within the context of a CG dimer, a potential hot spot for mutation (Barker et al., 1984).]

The conformer unique to FAP patient 3827 in exon 10 was found to contain a deletion of one nucleotide (1367, 1368, or 1369) when compared to the normal sequence found in the other bands on the SSCP gel. This deletion, occurring within a set of three T's, changed the sequence from CTTTCA to CTTCA; this 1 base frameshift creates a downstream stop within 30 bases. The PCR product amplified from this patient's DNA also was electrophoresed on an acrylamide-urea denaturing gel, along with the PCR product from a control DNA and products from a sequencing reaction. The patient's PCR product showed two bands differing by 1 bp in length, with the larger identical in mobility to the PCR product from the normal DNA; this result confirmed the presence of a 1 bp deletion in patient 3827.

Sequence analysis of the variant conformer of exon 11 from patient 3712 revealed the substitution of a T by a G at position changing the normal tyrosine codon to a stop codon.

The pair of conformers observed in exon 15 of the DP2.5 gene for FAP patient 3751 also was sequenced. These conformers were found to carry a nucleotide substitution of C to G at position 5253, the third base of a valine codon. No amino acid change resulted from this substitution, suggesting that this conformer reflects a genetically silent polymorphism.

The observation of distinct inactivating mutations in the DP2.5 gene in four unrelated patients strongly suggested that DP2.5 is the gene involved in FAP. These mutations are summarized in Table IIA.

#### EXAMPLE 10

This example demonstrates that the mutations identified in the DP2.5 (APC) gene segregate with the FAP phenotype.

Patient 3746, described above as carrying an APC allele with a frameshift mutation, is an affected offspring of two

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normal parents. Colonoscopy revealed no polyps in either parent nor among the patient's three siblings.

202.919 5 [DNA samples from both parents, from the patient's wife, and from their three children were examined. SSCP analysis of DNA from both of the patient's parents displayed the normal pattern of conformers for exon 7, as did DNA from the patients' wife and one of his off-spring. The two other children, however, displayed the same new conformers as their affected father. Testing of the patient and his parents 10 with highly polymorphic VNTR (variable number of tandem repeat) markers showed a 99.98% likelihood that they are his biological parents.]

15 These observations confirmed that this novel conformer, known to reflect a 2 bp deletion mutation in the DP2.5 gene, appeared spontaneously with FAP in this pedigree and was transmitted to two of the children of the affected individual.

#### EXAMPLE 11

20 This example demonstrates polymorphisms in the APC gene which appear to be unrelated to disease (FAP).

Sequencing of variant conformers found among controls as well as individuals with APC has revealed the following polymorphisms in the APC gene: first, in exon 11, at position 25 1458, a substitution of T to C creating an RsaI restriction site but no amino acid change; and second, in exon 15, at positions 5037 and 5271, substitutions of A to G and G to T, respectively, neither resulting in amino acid substitutions. These nucleotide polymorphisms in the APC gene sequence 30 may be useful for diagnostic purposes.

#### EXAMPLE 12

This example shows the structure of the APC gene.

35 The structure of the APC gene is schematically shown in FIG. 8, with flanking intron sequences indicated (SEQ ID NO:11-38).

200.27 [The continuity of the very large (6.5 kb), most 3' exon in DP2.5 was shown in two ways. First, inverse PCR with 40 primers spanning the entire length of this exon revealed no divergence of the cDNA sequence from the genomic sequence. Second, PCR amplification with converging primers placed at intervals along the exon generated products of the same size whether amplified from the originally isolated cDNA, cDNA from various tissues, or genomic template. 45 Two forms of exon 9 were found in DP2.5: one is the complete exon; and the other, labeled exon 9A, is the result of a splice into the interior of the exon that deletes bases 934 to 1236 in the mRNA and removes 101 amino acids from the predicted protein (see FIG. 3, SEQ ID NO:1 and 2).] 50

#### EXAMPLE 13

This example demonstrates the mapping of the FAP deletions with respect to the APC exons.

55 Somatic cell hybrids carrying the segregated chromosomes 5 from the 100 kb (HHW1291) and 260 kb (HHW1155) deletion patients were used to determine the distribution of the APC genes exons across the deletions. DNAs from these cell lines were used as template, along 60 with genomic DNA from a normal control, for PCR-based amplification of the APC exons.

PCR analysis of the hybrids from the 260 kb deletion of patient 3214 showed that all but one (exon 1) of the APC exons are removed by this deletion. PCR analysis of the 65 somatic cell hybrid HHW1291, carrying the chromosome 5 homolog with the 100 kb deletion from patient 3824, revealed that exons 1 through 9 are present but exons 10

through 15 are missing. This result placed the deletion breakpoint either between exons 9 and 10 or within exon 10.

## EXAMPLE 14

This example demonstrates the expression of alternately spliced APC messenger in normal tissues and in cancer cell lines.

Tissues that express the APC gene were identified by PCR amplification of cDNA made to mRNA with primers located within adjacent APC exons. In addition, PCR primers that flank the alternatively spliced exon 9 were chosen so that the expression pattern of both splice forms could be assessed. All tissue types tested (brain, lung, aorta, spleen, heart, kidney, liver, stomach, placenta, and colonic mucosa) and cultured cell lines (lymphoblasts, HL60, and choriocarcinoma) expressed both splice forms of the APC gene. We note, however, that expression by lymphocytes normally residing in some tissues, including colon, prevents unequivocal assessment of expression. The large mRNA, containing the complete exon 9 rather than only exon 9A, appears to be the more abundant message.

Northern analysis of poly(A)-selected RNA from lymphoblasts revealed a single band of approximately 10 kb, consistent with the size of the sequenced cDNA.

## EXAMPLE 15

This example discusses structural features of the APC protein predicted from the sequence.

*See APP 13* The cDNA consensus sequence of APC predicts that the longer, more abundant form of the message codes for a 2842 or 2844 amino acid peptide with a mass of 311.8 kd. This predicted APC peptide was compared with the current data bases of protein and DNA sequences using both Intelligenetics and GCG software packages. No genes with a high degree of amino acid sequence similarity were found. Although many short (approximately 20 amino acid) regions of sequence similarity were uncovered, none was sufficiently strong to reveal which, if any, might represent functional homology. Interestingly, multiple similarities to myosins and keratins did appear. The APC gene also was scanned for sequence motifs of known function; although

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multiple glycosylation, phosphorylation, and myristoylation sites were seen, their significance is uncertain.]

- 5 Analysis of the APC peptide sequence did identify features important in considering potential protein structure. Hydropathy plots (Kyte and Doolittle, J. Mol. Biol. Vol. 157, pp. 105-132 (1982)) indicate that the APC protein is notably hydrophilic. No hydrophobic domains suggesting a signal
- 10 peptide or a membrane-spanning domain were found. Analysis of the first 1000 residues indicates that  $\alpha$ -helical rods may form (Cohen and Parry, Trends Biochem. Sci. Vol. 77, pp. 245-248 (1986); there is a scarcity of proline residues and, there are a number of regions containing
- 15 heptad repeats (apolar-X-X-apolar-X-X-X). Interestingly, in exon 9A, the deleted form of exon 9, two heptad repeat regions are reconnected in the proper heptad repeat frame, deleting the intervening peptide region. After the first 1000
- 20 residues, the high proline content of the remainder of the peptide suggests a compact rather than a rod-like structure.

The most prominent feature of the second 1000 residues is a 20 amino acid repeat that is iterated seven times with

25 semiregular spacing (Table 4). The intervening sequences between the seven repeat regions contained 114, 116, 151, 205, 107, and 58 amino acids, respectively. Finally, residues 2200-24000 contain a 200 amino acid basic domain.

30 [TABLE IV

Seven Different Versions of the 20-Amino Acid Repeat

Consensus:	F*VE*TP*CFSR*SSLSSLS
1262:	YCVEDTPICFSRCSSLSLS
1376:	HTVQETPLMFSCRCTSVSSLD
1492:	FATESTPDGFCSSLSALS
1643:	YCVEGTPINFSTATSLSDLT
1848:	TPIEGTPYCFSRNDSLSSLD
1953:	FAIENTPVCPSHNSSLSSLS
2013:	RHVEDTPVCFSRNSSLSSLS

40 Numbers denote the first amino acid of each repeat. The consensus sequence at the top reflects a majority amino acid at a given position.]

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